Epidermolysis Bullosa Acquisita Antigen: Relationship Between the Collagenase-Sensitive and -Insensitive Domains

Takashi Yoshiike, M.D., David T. Woodley, M.D., Robert A. Briggaman, M.D.
Department of Dermatology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina, U.S.A.

To clarify the relationship of the 290 and 145 kDa chains of the epidermolysis bullosa acquisita (EBA) antigen, we subjected urea extracts of skin basement membrane zone (BMZ) proteins and isolated 290 and 145 kDa chains of the EBA antigen cut out of sodium dodecyl sulfate polyacrylamide gels to treatment with clostridial collagenase. When the reaction products were electrophoresed, transblotted, and reacted with EBA patient sera or two monoclonal antibodies to the EBA antigen, the 290 kDa chain was degraded into the 145 kDa band that was resistant to cleavage with collagenase. The 145 kDa domain, isolated after collagenase treatment of the whole BMZ extract, was resistant to degradation by hyaluronidase, chondroitinase ABC, heparinase, and heparitinase but was readily degraded by V-8 protease. These data suggest that the EBA antigen consists of collagen and noncollagen domains of identical size (M_r 145,000), and that the 145 kDa noncollagen domain is generated via degradation of the native 290 kDa species by collagenase. *(J Invest Dermatol 90:127–133, 1988)*

Epidermolysis bullosa acquisita (EBA) is a serious acquired subepidermal bullous disease [1] that has also been called "dermolytic pemphigoid" [2]. Patients with EBA have an anti-basement zone autoantibody deposited within the dermal-epidermal junction of their skin and often circulating in their serum [3–7]. These antibodies bind to the interface of the lamina densa and sublamina densa zone in the cutaneous basement membrane [8,9]. The target for these antibodies is a large matrix molecule that is recognized by Western blot analysis of human skin basement membrane proteins as 290 and 145 dKa chains [8]. Only the 290 kDa chain has been immunoprecipitated from labeled human cell cultures [10–12]. We have reported previously [13] that the 290 kDa chain is sensitive to collagenase and that both the 290 and 145 kDa bands are degraded by V-8 protease. However, until now the relationship between the 290 and 145 kDa chains has been unclear. In this paper, we introduce a new monoclonal antibody, EBA-2, to the EBA antigen and extend our previous work by showing conclusively that the 145 kDa chain is generated by collagenase treatment of the 290 kDa chain and that the 145 kDa chain of the antigen is collagenase-insensitive. These data indicate that the EBA antigen consists of a 145 kDa collagen domain and a 145 kDa noncollagen domain that are covalently linked to form the high molecular weight 290 kDa chain seen by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting.

Manuscript received February 26, 1987; accepted for publication July 3, 1987.
This investigation was supported by grants AM01540, AM33625, and AM10546 from the National Institutes of Health.
Reprint requests and correspondence to: David T. Woodley, M.D., Department of Dermatology, University of North Carolina, School of Medicine, Chapel Hill, NC 27514.

Abbreviations:
BMZ: basement membrane zone
BSA: bovine serum albumin
EBA: epidermolysis bullosa acquisita
EDTA: ethylenediamine tetraacetate
kDa: kilodaltons
M_r: molecular weights
NEM: *N*-ethylmaleimide
PBS: phosphate-buffered saline
PMSF: phenylmethyl sulfonyl fluoride
SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

MATERIALS AND METHODS

Preparation of Basement Membrane Adult human skin basement membrane zone proteins were prepared as previously described [8] by extraction in 8 M urea, 0.3 M beta-mercaptoethanol, and 0.05 M Tris-HCl (extraction buffer), except that protease inhibitors were added to the urea extraction buffer and the pH lowered to pH 7.5. The protease inhibitors added to the extraction buffer were 0.001 M phenylmethylsulfonyl fluoride (PMSF), 0.002 M *N*-ethylmaleimide (NEM), and 0.001 M ethylenediamine tetraacetate (EDTA).

Epidermolysis Bullosa Acquisita Antibodies Human polyclonal antibodies were collected from three patients with active EBA. These antibodies localized to the epidermal–dermal junction of human skin by immunofluorescence and to both the sublamina densa and lamina densa regions by immunoelectron microscopy. A murine monoclonal antibody, previously designated H_3a [14] and now termed EBA-1 in this paper, was also used.

A second new monoclonal antibody designated EBA-2 was prepared through immunization of BALB/c mice by intraperitoneal injection of 2 mg of basement membrane zone extract in 0.25 ml phosphate-buffered saline (PBS) homogenized with an equal volume of complete Freund's adjuvant. Following booster immunizations approximately 4 wk later, spleens were harvested and the cells fused with nonsecreting P3/NS1 mouse myeloma cells as previously described [14]. Culture supernates were screened for basement membrane antibodies by indirect immunofluorescence and cultures of interest cloned by limiting dilution. The EBA-2 monoclonal antibody was selected by its intense reactivity with the human skin basement membrane zone. This localization was identical to that seen with the EBA-1 (H_3a) mouse monoclonal anti-

0022-202X/88/$03.50

body. However, EBA-2 had different physical properties since it reacted with staphylococcal protein A and complement, whereas the previously described EBA-1 did not.

Immunoelectron Microscopy Immunoelectron microscopy was performed as previously described using a multistep immunoperoxidase procedure [8,14]. In these experiments, the secondary antibody was either a goat antimouse IgG or a goat antihuman IgG, depending on whether polyclonal human or monoclonal mouse antibodies were used. Controls included normal goat serum, normal human serum, culture medium used for monoclonal cultures with 20% fetal calf serum and PBS.

Polyacrylamide Gels and Western Immunoblots Human skin BMZ proteins in the extract were separated by SDS PAGE, transferred electrophoretically to nitrocellulose paper and reacted with experimental antibodies to the EBA antigen (human polyclonal antibodies or the murine monoclonal antibodies, EBA-1 and EBA-2) or control solutions (normal human serum, or a monoclonal antibody to fibronectin) as previously described [8,14–16].

In other experiments, the 290 and 145 kDa EBA chains were identified in Coomassie blue–stained gels, cut out of the gels, re-electrophoresed in a second identical gel and subjected to Western blotting as previously described [13].

Collagenase Treatment of Skin BMZ Proteins Four hundred micrograms of lyophilized BMZ extract were solubilized in 180 μl of extraction buffer and dialyzed against 0.025 M Tris-HCl, 0.01 M calcium acetate, 0.001 M PMSF, 0.04 M NEM, 5 μg/ml pepstatin A, pH 7.2 (collagenase buffer), at 4°C. Four units (20 μl) of proteinase-free collagenase (Type III; Advance Biofactures, Lynbrook, New York) were added and incubated at 37°C for 5, 10, and 30 min, and 1, 6, and 12 h. In some experiments, a more thorough digestion was performed by using more collagenase (100 U) and a longer incubation time (48 h). Identical parallel controls (0.5–48 h), to which the same amounts of boiled collagenase were added, were prepared. Another 48-h control was performed with collagenase in the presence of 0.010 M (EDTA). The collagenase activity was stopped by placing the tubes on ice and adding 20 μl of 0.1 M EDTA. The samples were then dialyzed against 0.025 M sodium phosphate (pH 6.8) 2% SDS, 10% glycerol, 0.002% bromophenol blue (sample buffer), and made 0.1 M with dithrotreitol, boiled for 2 min, and subjected to SDS PAGE and Western blot analysis.

To determine that the collagenase enzyme and the conditions of the experiments were specific for collagen degradation and would not degrade noncollagen proteins, parallel control studies were performed under the same conditions with human skin type I collagen [17], laminin [17], immunoglobulin (Miles Laboratories, Elkhart, Indiana), bovine serum albumin (BSA) (Sigma Chemical Co, St Louis, Missouri), and fibronectin [17]. In these experiments, however, 16 U of protease-free collagenase were added to each tube containing 400 μg of protein and incubated at 37°C for 36 h. After the reactions were terminated by cooling and the addition of EDTA as above, the samples were dialyzed into sample buffer and subjected to SDS PAGE.

Collagenase Treatment of Isolated EBA Antigen Chains SDS PAGE was performed on crude human skin BMZ protein, type I collagen, and BSA and the gels stained with Coomassie Blue [14]. The 290 and 145 kDa EBA antigen chains, alpha 1 chains of type I collagen (M_r 98,000), and BSA monomers (M_r 66,000) were identified, cut out of the gels [13,18]; and dried with a hair dryer for 30 min. Two dried bands of each material (approximately 80 to 120 μg of protein) were placed into collagenase buffer containing 100 U of unboiled or boiled collagenase and incubated at 37°C for 48 h. The reactions were terminated by adding 40 μl of 0.1 M EDTA and cooling the samples on ice. After the collagenase treatment, the bands were equilibrated and the collagenase solutions dialyzed against sample buffer. The dialysates were concentrated to 100 μl, dithiothreitol was added to 0.1 M, and the samples boiled for 2 min. The solid gel bands and their respective proteins solubilized in collagenase solution were then placed together into a well of an identical second slab gel, re-electrophoresed and immunoblotted. To determine EBA antigen products that resulted from collagenase digestion, we reacted the blots with EBA antibodies. As a positive control for collagenase, the blots of the treated collagen bands were reacted with anti-type I collagen antibodies [17]. The blots of the treated albumin bands reacted with antiserum bovine albumin antibodies served as negative controls.

Treatment of Collagenase-Treated EBA Antigen With Other Enzymes One-hour collagenase treated BMZ proteins as described above, were subjected to further treatment with hyaluronidase, chondroitinase ABC (Advanced Biofactures, Lynbrook, New York), heparinase, heparitinase; and staphylococcal V8 protease (ICN Immunologicals, Lisle, Illinois).

Two hundred microliters of collagenase treated BMZ protein (400 μg) were dialyzed against 0.1 M sodium acetate, pH 6.0 (hyaluronidase buffer), 0.05 M Tris-acetate, pH 8.0 (chondroitinase ABC buffer), 0.1 M sodium acetate, pH 7.0, 3.3 mM calcium acetate (heparinase and heparitinase buffer), and 0.05 M Tris-acetate, pH 7.8 (V-8 protease buffer). After dialysis, 20 U of hyaluronidase, 0.4 U of chondroitinase ABC, 4 U of heparinase, 4 U of heparitinase, and 5 μg of V-8 protease in the volume of 20 μl were added and then incubated for 2 h at 43°C (hyaluronidase and heparitinase), 37°C (chrondroitinase ABC and V-8 protease), or 35°C (heparinase). Boiled enzymes were used as controls. Reaction mixtures were then dialyzed against SDS PAGE sample buffer with 0.1 M dithiothreitol, boiled for 2 min, and subjected to SDS PAGE and Western blot analysis.

Immunoprecipitation of Proline-Labeled EBA Antigen The collagenase digestion conditions described were specific for the degradation of collagen (vide infra, Results and Fig 3). However, to determine further that the EBA antigen is collagenous in nature, human skin fibroblast cultures were labeled with [^{3}H]proline, and the 290 kDa EBA antigen was specifically immunoprecipitated from extracts of the cell layers with antibodies from the sera of EBA patients as previously described [10,12]. Briefly, human skin fibroblasts were grown to 90% confluency in Dulbecco's minimal essential medium (DMEM) with 10% fetal calf serum (Gibco Laboratories, Grand Island, New York). The medium was then changed to DMEM with 20% dialyzed fetal bovine serum (Gibco #220-6300, Grand Island, New York) and 50 μg/ml of ascorbic acid (Sigma), and the cultures incubated at 37°C for 3 h. Then 20 μCi/ml of L-2,3,4,5-tritiated proline (TRK 534; Amersham, Arlington Heights, Illinois) was added and the cultures incubated overnight at 37°C in a humidified incubator with 5% CO_2. The medium was collected and the cell layers washed with fresh medium 3 times. The cell layers were extracted in 0.01 M Tris-HCl, 0.25 M NaCl (pH 7.4), 0.3% Nonidet P-40, 0.3% sodium deoxycholate, 0.001 M PMSF, 0.001 M EDTA, 0.1% bovine serum albumin by manually scraping the cell layers and collecting the cells and buffer. The proline-labeled proteins in the extract were immunoprecipitated with two EBA sera [8], two normal human sera, an affinity-purified goat anti-type I collagen antibody [17], or normal goat serum as previously described [10,12].

RESULTS

Antibody Labeling of EBA Antigen/Comparison of Monoclonal and Polyclonal EBA Antibodies Using immunoelectron microscopy, 7 human polyclonal sera derived from patients with EBA were compared with two mouse monoclonal antibodies, EBA-1 and EBA-2. All of the polyclonal EBA sera localized antigens in a more extensive distribution at the epidermal–dermal junction than the monoclonal antibodies. The polyclonal EBA antibodies were localized to the lamina densa and sublamina densa regions, where dense, uniformly distributed immune reaction products were seen throughout the entire region (Fig 1). On the other hand, both mouse monoclonal antibodies recognized antigens predominately in the lamina densa. Immune reaction products were densely distributed over the entire lamina densa, with only scattered

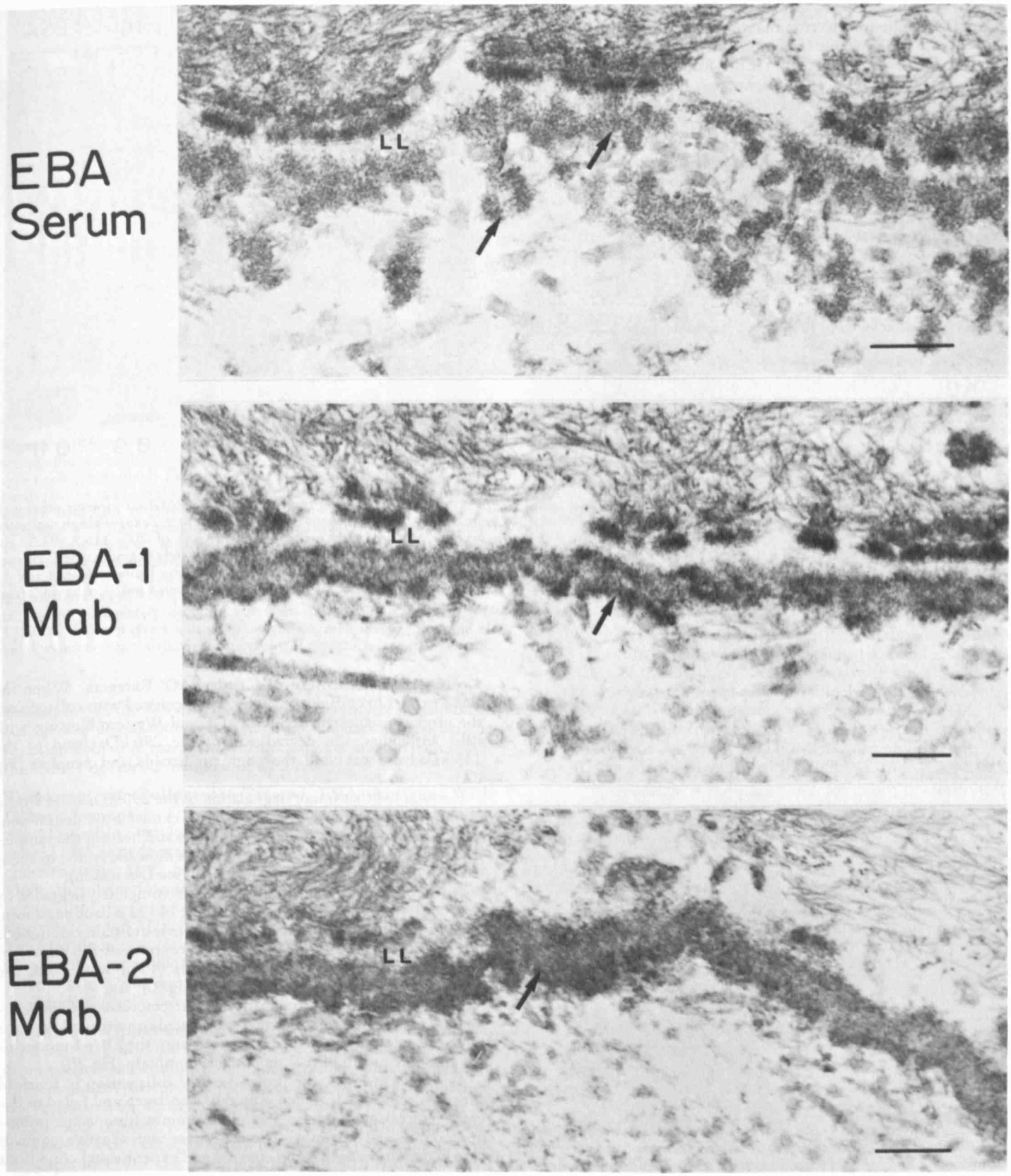

Figure 1. Immunoelectron micrographs using polyclonal and monoclonal EBA antibodies on normal human skin substrate. The upper panel (EBA serum) shows immune deposits *(arrows)* associated with EBA antigen in a broad distribution overlapping the lamina densa and sublamina densa. The lamina lucida *(LL)* is free of deposits. The middle (EBA-1 Mab) and lower (EBA-2 Mab) panels show immune deposits *(arrow)* localized predominantly to the lamina densa. The lamina lucida *(LL)* is free of deposits. These observations indicate that the monoclonal EBA antibodies recognize antigenic determinant(s) on a domain of the EBA antigen restricted to the lamina densa. ×60,000. *Bar = 0.25 μm.*

areas of dense reaction products in the sublamina densa area (Fig 1). Like EBA-1, immunoelectron microscopy of human skin substrate showed that the antigenic target for EBA-2 was localized predominately to the lamina densa region. EBA-1 monoclonal antibody and antibodies in the sera of EBA patients have been well characterized previously [4,8,12,14] and are identical to EBA-2 by indirect immunoelectron microscopy (Fig 1) and Western blot analysis (Fig 2). The labeled 290 kDa EBA antigen bands have been shown previously to be distinct from other known matrix molecules [8]. Unlike EBA-1 monoclonal antibody, EBA-2 antibody bound readily to staphyloccal protein A and complement.

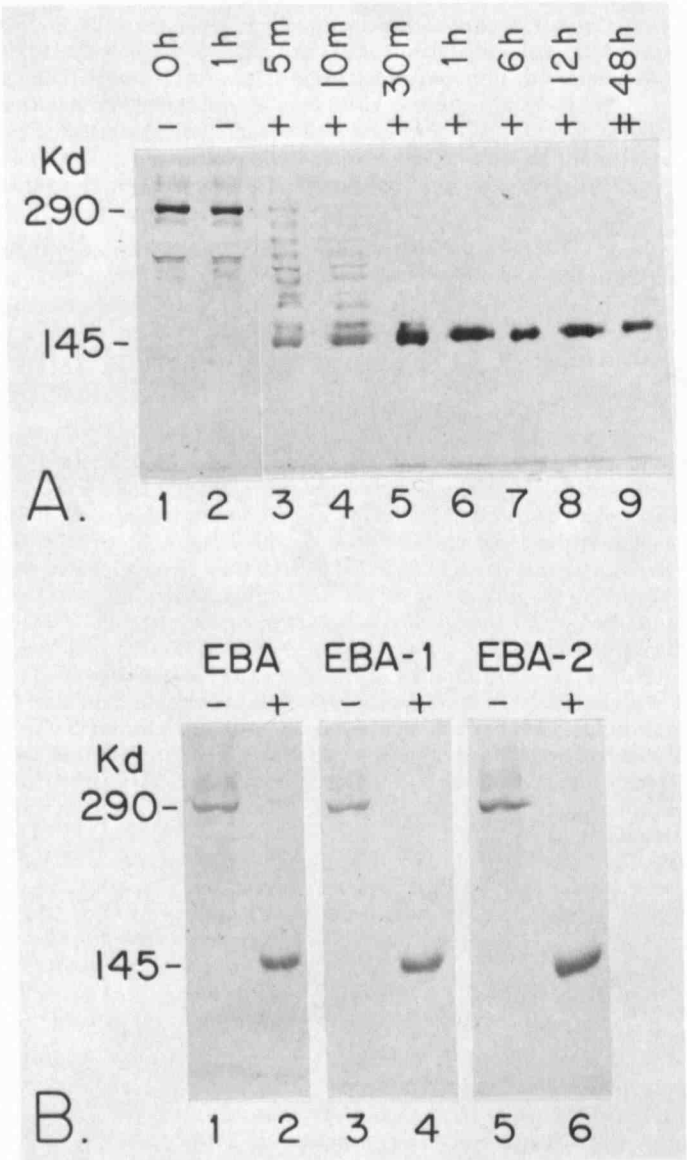

Figure 2. *A*, Western blot of basement membrane zone extract incubated with 4 units of active collagenase (+) or inactive boiled collagenase (−) for the times indicated and the products reacted with EBA antibodies. Degradation of the 290 kDa EBA antigen domain to the 145 kDa domain was rapid and complete by 1 h *(lane 6)*. Prolonged incubations of 48 h with 100 U of active collagenase (++) did not induce degradation of the 145 kDa EBA antigen domain *(lane 9)*. *B*, Western blot of basement membrane zone extracted and incubated with 4 U of active collagenase (+) or inactive boiled collagenase (−) for 1 h and reacted with a 1:400 dilution of serum from a patient with EBA *(lanes 1* and *2)*, a monoclonal antibody, EBA-1, to the EBA antigen *(lanes 3* and *4* [see reference 13]), and a new monoclonal antibody, EBA-2, to the EBA antigen *(lanes 5* and *6)*.

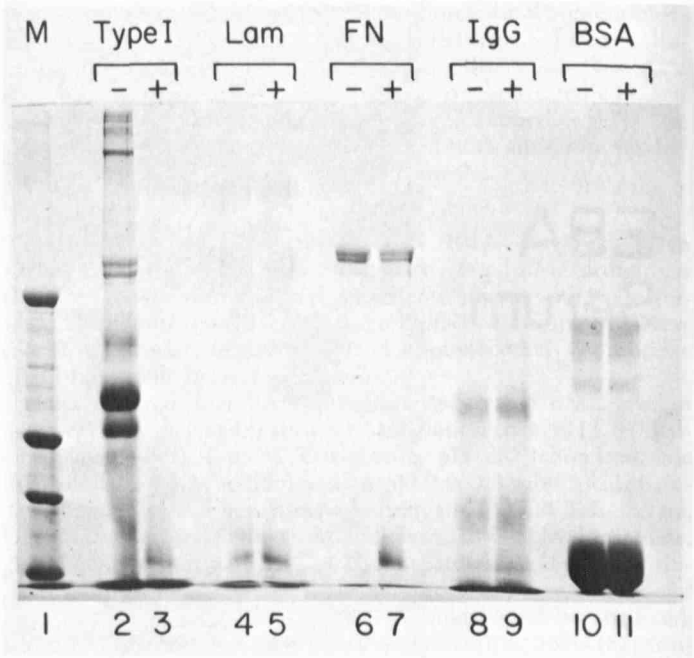

Figure 3. A Coomassie Blue–stained SDS PAGE of proteins previously treated with (+) or without (−) active collagenase. *Lane 1* is a high molecular weight standard with major bands (arrows) of 200, 116.2, 92.5, and 66.2 kDa. Notice that the collagenase completely degrades type I collagen (compare *lanes 2* and *3*), but has no effect upon laminin *(lanes 4* and *5)*, fibronectin *(lanes 6* and *7)*, immunoglobulin *(lanes 8* and *9)*, or bovine serum albumin *(lanes 10* and *11)*.

Collagenase Treatment of Crude BMZ Extracts When the BMZ extract in collagenase buffer was incubated with collagenase, the products subjected to SDS PAGE and Western blotting with EBA antibodies, the degradation of the 290 kDa band to the 145 kDa band was rapid, thorough, predictable, and complete (Fig 2*A*).

It was noted that minor degradation of the 290 kDa band would often occur simply by dialyzing the BMZ extract into calcium-containing buffer without protease inhibitors and heating the samples to 37°C (Fig 2*A*, *lanes 1* and *2*). This was most likely due to coextraction of skin mammalian collagenase (see Discussion).

Further, although the 290 kDa band was completely degraded by the addition of extrinsic collagenase into a 145 kDa band at 30 min, the 145 kDa band itself remained collagenase-resistant throughout the 12-h assay. Even when higher concentrations (20 times) of collagenase and longer incubation times (48 h) were used, the 145 kDa band showed no degradation (Fig 2*A*, *lane 9*).

Identical Western blotting patterns were observed (with or without collagenase treatment) when the treated or untreated proteins were labeled with the sera from EBA patients, the EBA-1 monoclonal antibody or the EBA-2 monoclonal antibody (Fig 2*B*).

As shown in Fig 3, the proteinase-free collagenase, in fourfold excess, specifically degraded only type I collagen and failed to degrade noncollagen proteins, including laminin, fibronectin, immunoglobulin, and albumin. These positive and negative controls clearly demonstrate that the enzyme and experimental conditions were specific for collagen degradation.

Collagenase Treatment of Isolated EBA Antigen Chain The cut out protein bands were incubated with collagenase for 48 h and the products were electrophoresed, transferred to nitrocellulose paper, and reacted with corresponding antibodies. The 290 kDa EBA antigen chain was readily degraded into the 145 kDa chain when incubated with collagenase (Fig 4, *lanes 5–8*). However, even with high collagenase concentrations (500 U/ml) and 48-h incuba-

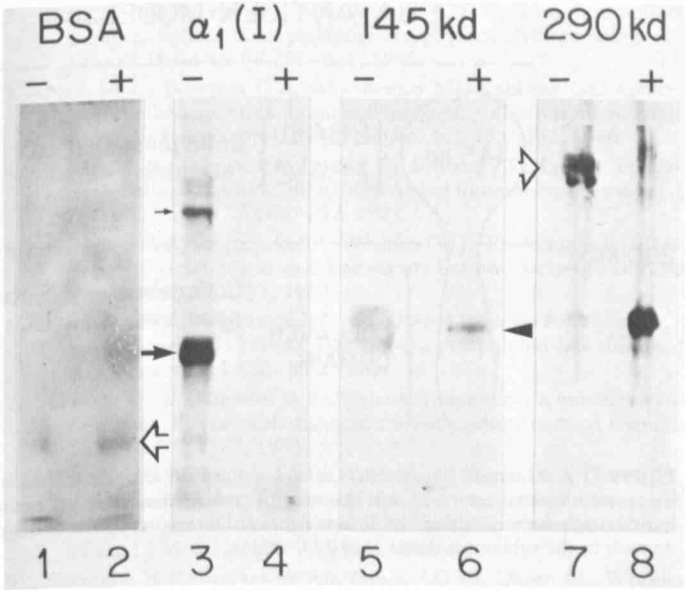

Figure 4. Western blots of bovine serum albumin reacted with antibovine serum albumin antibody *(lanes 1* and *2)*, type I collagen reacted with anti-type I collagen antibody *(lanes 3* and *4)*, and isolated 145 kDa EBA antigen bands *(lanes 5* and *6)* or isolated 290 kDa EBA antigen bands *(lanes 7* and *8)* reacted with EBA antibodies *(lanes 5–8)*. All of the substrates have been incubated with 100 U of active collagenase (+) or boiled inactive collagenase (–). In accordance with the whole extract experiments, the 145 kDa EBA antigen domain *(solid arrow without tail)* is resistant to degradation by collagenase *(lane 6)*, while the 290 kDa domain *(hollow arrow without tail)* is degraded to the 145 kDa species *(lanes 7* and *8)*. *Lanes 3* and *4* show that the collagenase completely degrades the alpha (M_r 95,000) and beta (M_r 195,000) chains of type I collagen *(short* and *long solid arrows; lanes 3* and *4)* but does not degrade bovine serum albumin (M_r 66,000, *hollow arrow, lane 2*).

tions at 37°C, the 145 kDa band remained completely resistant to collagenase degradation. The results were identical whether the immunoblots were reacted with EBA patients' sera or with either the EBA-1 or EBA-2 monoclonal antibody.

Treatment of Collagenase-Treated EBA Antigen With Other Enzymes To characterize the collagenase resistant 145 kDa chain, we further subjected thoroughly collagenase-treated EBA antigen preparations to treatment with hyaluronidase, chondroitinase ABC, heparinase, heparitinase, and V8 protease. Under these experimental conditions, the 145 kDa chain was resistant to all of the enzymes except V-8 protease, which completely degraded the 145 kDa chain. Although the staining intensity of the 145 kDa chain was decreased by both hyaluronidase digestion and its control, it is possible that the antigenic epitope was partly denatured by the acidic and high temperature conditions used in the experiments (Fig 5, *lanes 1* and *2*).

Immunoprecipitation of Proline-Labeled EBA Antigen The 290 kDa EBA antigen was specifically immunoprecipitated from proline-labeled human fibroblast cultures with antibodies in the sera of EBA patients (Fig 6). Antibody to type I collagen immunoprecipitated the alpha 2 and alpha 1 chains of type I collagen (M_r 98,000 and 105,000, respectively) but not the EBA antigen. The antibody to type I collagen served not only as a positive control for the experiment but also confirmed that the EBA antigen was not related to type I collagen. Normal goat and human serum did not immunoprecipitate any specific proline-labeled proteins. It should be noted that only the 290 kDa species of the EBA antigen was immunoprecipitated from the cultures and not the 145 kDa domain. This again suggests that the 290 kDa species is the native EBA antigen. While collagens are rich in proline, other proteins usually contain minimal amounts of this amino acid. The fact that the 290 kDa EBA antigen could be immunoprecipitated from proline-labeled cultures further supports the collagenous nature of the EBA antigen and is in accordance with the collagenase data.

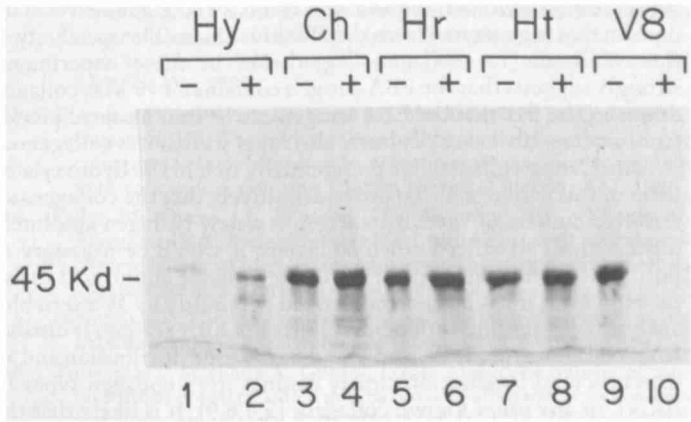

Figure 5. Western blot reacted with EBA antibody of the 145 kDa EBA antigen domain generated by collagenase treatment of the whole basement membrane zone extract and then further incubated with a second active enzyme (+) or inactive control (–), including hyaluronidase *(lanes 1* and *2)*, chondroitinase *(lanes 3* and *4)*, heparinase *(lanes 5* and *6)*, heparitinase *(lanes 7* and *8)*, or V-8 protease *(lanes 9* and *10)*. Only the V-8 protease degrades the 145 kDa domain. The poor antibody labeling seen with *both* the control and with hyaluronidase is likely due to the acidic conditions and high temperature conditions used with this particular enzyme.

DISCUSSION

The EBA antigen was recently identified as a distinct BMZ component consisting of two different chains of 290 and 145 kDa [8]. The insolubility of the EBA antigen, the small amounts in skin, and the limited quantities of human skin have hampered detailed study and purification of this molecule. Previous analytical studies with V-8 protease mapping suggested some heterogeneity of the two chains [13]. Although both chains contained carbohydrate, the 145 kDa band contained more than the 290 kDa band [13]. Despite the biochemical differences in the two EBA chains, antibodies in both patients' sera and the monoclonal antibodies (EBA-1, EBA-2) label both chains in Western blots. However, until now the relationship between the two chains was unclear. Collagenase treatment of both crude skin BMZ extracts and isolated EBA antigen chains in this

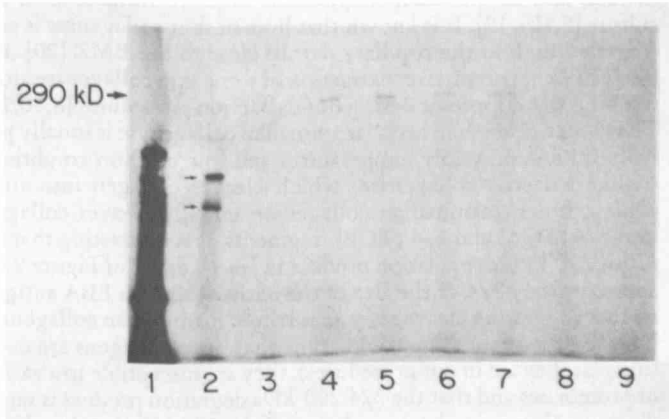

Figure 6. Biosynthetically labeled EBA antigen immunoprecipitated from proline-labeled fibroblast cultures with EBA antibodies *(lanes 4–7)* but not with antibodies to type I collagen *(lane 2)*, normal goat serum *(lane 3)*, or normal human sera *(lanes 8* and *9)*. Note that the alpha chains of type I collagen *(small arrows)* are immunoprecipitated with antibody to type I collagen *(lane 2)*. Also note that only the 290 kDa EBA antigen species is immunoprecipitated and not the 145 kDa domain *(lanes 4–7)*. *Lane 1* is a high molecular weight standard, the highest band being 200 kDa.

study clearly shows that the 145 kDa chain is a collagenase-resistant domain that is generated from the 290 kDa chain. The specificity of the conditions for collagen degradation in these experiments strongly suggests that the EBA antigen contains a 145 kDa collagen domain. The fact that the EBA antigen can be immunoprecipitated from proline-labeled cell cultures also suggests that it is collagenous in nature, since collagens are preferentially rich in the hydroxylated form of this amino acid. To prove definitively that the collagenase-sensitive domain of the EBA antigen is a new collagen absolutely distinct from all other known collagens, it would be necessary to purify the EBA antigen and demonstrate a unique amino acid analysis. However, it has been demonstrated previously by Western blot analysis with specific antibodies [8] that the EBA antigen is distinct from collagen types I, IV, and V and has a tissue distribution and an ultrastructural localization clearly distinct from collagen types II, III, VI, or any other known collagens [3,4,8,9]. It is likely that the EBA antigen collagen domain is a new collagen limited to basement membrane beneath squamous epithelium. However, amino acid analysis of the purified molecule will be necessary to verify this hypothesis.

The studies reported here clarify the molecular organization of the EBA antigen. These data suggest that the 290 kDa chain identified in Western immunoblots labeled with EBA antibodies consists of collagen and noncollagen domains of equal size, 145 kDa each. The collagenase used was from *Clostridium histolyticum,* type III, substantially free of proteinase and peptidase activity. It cleaves the X-Gly bond of undenatured collagen chains into many small (pentapeptides) peptides [19]. This is in contrast to mammalian collagenase, which cleaves interstitial collagens into large three-quarter–one-quarter chain fragments [19–22]. The addition of extrinsic bacterial collagenase to the extract followed by incubation for 1–48 h completely obliterates the 290 kDa band, and produces no recognizable collagen degradation products (ie, intermediate between M_r 290,000 and 145,000) except the collagenase-insensitive 145 kDa domain. This suggests complete degradation of a 145 kDa collagen portion of the 290 kDa chain by the bacterial collagenase, leaving another noncollagen domain of equal size that is recognized in the blots by EBA antibodies. Thus, the model for the EBA antigen molecule is two 145 kDa domains covalently linked: one domain a collagen and the other equal-sized domain a noncollagen rich in carbohydrate [13].

As noted in *lanes 1* and *2* of Fig 2*A*, some minor degradation of the 290 kDa band may occur simply by dialyzing the BMZ extract into calcium-containing buffer without protease inhibitors and heating the samples to 37°C. The 145 kDa band has always been less intense and less consistently found in Western blots of the BMZ extracts and in immunoprecipitations of EBA antigen from cell culture [8,10–13]. It is known that human skin collagenase is concentrated high in the papillary dermis close to the BMZ [20]. It is likely that there is often coextraction of some skin collagenase along with the BMZ proteins during our extraction procedures [8,10,13]. The co-extracted "intrinsic" mammalian collagenase is usually partially inhibited by low temperatures and low calcium conditions. Unlike bacterial collagenase, which cleaves collagen into small pentapeptides, mammalian collagenase initially cleaves collagens into 3/4 (TCA) and 1/4 (TCB) fragments. It is interesting that the major 290 kDa degradation product in *lanes 1* and *2* of Figure 2*A* is approximately 3/4 of the size of the native 290 kDa EBA antigen, further suggesting cleavage by an intrinsic mammalian collagenase. On the other hand, one could argue that once collagens are denatured (as they are in our procedures), they are susceptible to a variety of proteinases and that the 3/4 290 kDa degration product is serendipitous. However, the degradation of the 290 kDa band under all conditions (Fig 2*A*, *lanes 1–9*) was inhibited by EDTA and not by PMSF or NEM.

Antibody binding sites for both of the mouse monoclonal antibodies are located on the 145 kDa domain of the EBA antigen as evidenced by the reactivity of these antibodies with the isolated 145 kDa protein and the 145 kDa domain prepared by collagenase digestion of the isolated 290 kDa protein. Monoclonal antibodies would be expected to recognize specific epitopes with restricted reactivity. For this reason, we believe that the mouse monoclonal antibodies recognize epitopes restricted to the 145 kDa domain, although we do not have direct evidence to exclude reactivity with the collagenous domain as well. Since the 145 kDa domain is a part of the 290 kDa EBA antigen molecule, we would expect the monoclonal antibodies to react with the 290 kDa molecule, This explains the reactivity of the monoclonal antibodies with both the 145 and 290 kDa EBA antigens described previously.

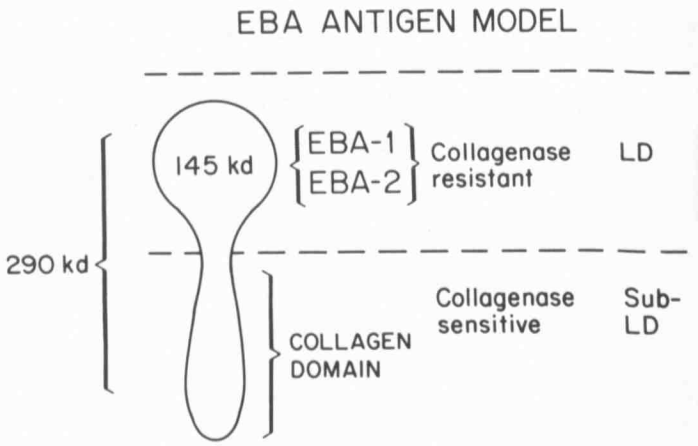

Figure 7. A schematic hypothetical model of the EBA antigen within the basement membrane zone of skin showing the collagenase-resistant carbohydrate-rich domain within the lamina densa *(LD)* region and the collagen domain in the sublamina densa *(Sub-LD)* region.

The human polyclonal antibodies derived from the sera of patients with EBA also react with the isolated 145 kDa band and the 145 kDa domain prepared by collagenase digestion of the isolated 290 kDa protein. These polyclonal antibodies may also react with other epitopes on the collagenase-sensitive domain in addition to the epitopes recognized on the 145 kDa noncollagenous domain. This is suggested by the demonstrated differences in the ultrastructural localization of the monoclonal antibodies and the polyclonal antibodies from EBA patients. The polyclonal antibodies have a more extensive distribution at the epidermal–dermal junction by immunoelectron microscopy than do the monoclonal antibodies. The polyclonal EBA antibodies are distributed in the lamina densa area as well as in the sublamina densa area. On the other hand, both mouse monoclonal antibodies recognize antigens predominantly localized in the lamina densa (Fig 1).

The immunoelectron microscopic data taken together with the biochemical data would suggest that the EBA antigen is composed of a 145 kDa noncollagenous domain localized in the lamina densa and a collagenous domain distributed in the sublamina densa region (Fig 7). It is likely that the native form of the EBA antigen is the 290 kDa chain because it is more intensely stained and more consistently found in Western blots than the 145 kDa chain and is the only species of the EBA antigen immunoprecipitated from cell culture [10–12]. The data further suggest that the 290 kDa chain consists of collagen and noncollagen domains of identical molecular weight. The collagenous domain is labile and may be partly or completely degraded by collagenase. Antigenic determinants for monoclonal and polyclonal antibody are in the carbohydrate-rich noncollagen domain.

The authors are grateful to Ms. Melinda J. Reese for her technical assistance and to Ms. Donna Foushee for preparing the manuscript.

REFERENCES

1. Roenig HH Jr, Ryan JG, Bergfeld WF: Epidermolysis bullosa acquisita: report of three cases and review of all published cases. Arch Dermatol 103:1–10, 1971

2. Briggaman RA, Gammon WR, Woodley DT: Epidermolysis bullosa acquisita of the immunopathological type (dermolytic pemphigoid). J Invest Dermatol 85:795–845, 1985
3. Nieboer C, Boorsma DM, Woerdeman MJ, Kalsbeek GL: Epidermolysis bullosa acquisita: immunofluorescence, electron microscopic studies in four patients. Br J Dermatol 102:383–392, 1980
4. Yaoita H, Briggaman RA, Lawley TJ, Provost TT, Katz SI: Epidermolysis bullosa acquisita: ultrastructural and immunological studies. J Invest Dermatol 76:288–292, 1981
5. Gammon WR, Briggaman RA, Wheeler CE Jr: Epidermolysis bullosa acquisita presenting as an inflammatory bullous disease. J Am Acad Dermatol 7:382–387, 1982
6. Gammon WR, Briggaman RA, Woodley DT, Heald P, Wheeler CE Jr: Epidermolysis bullosa acquisita—a pemphigoid-like disease. J Am Dermatol 11:820–832, 1984
7. Woodley DT, Gammon WR: Epidermolysis bullosa acquisita: an autoimmune disease with distinctive immunoultrastructural features. Cutis 32:521–527, 1983
8. Woodley DT, Briggaman RA, O'Keefe EJ, Inman AO, Queen LL, Gammon WR: Identification of the epidermolysis bullosa acquisita antigen—a normal component of human skin basement membrane. N Engl J Med 310:1007–1013, 1984
9. Gammon WR, Briggaman RA, Inman AO III, Queen LL, Wheeler CE Jr: Differentiating anti-lamina lucida and anti-sublamina densa anti-BMZ antibodies by indirect immunofluorescence on 1.0 M sodium chloride-separated skin. J Invest Dermatol 82:139–144, 1984
10. Woodley DT, Briggaman RA, Gammon WR, O'Keefe EJ: Epidermolysis bullosa acquisita antigen is synthesized by human keratinocytes cultured in serum-free medium. Biochem Biophys Res Commun 130:1267–1272, 1985
11. Stanley JR, Rubinstein N, Klaus-Kovtun V: Epidermolysis bullosa acquisita antigen is synthesized by both human keratinocytes and human dermal fibroblasts. J Invest Dermatol 85:542–545, 1985
12. Woodley DT, Briggaman RA, Gammon WR, Falk RJ, Reese MJ, Tomsick RS, O'Keefe EJ: Epidermolysis bullosa acquisita antigen, a major cutaneous basement membrane, is synthesized by human dermal fibroblasts and other cutaneous tissues. J Invest Dermatol 87:227–231, 1986
13. Woodley DT, O'Keefe EJ, Reese MJ, Mechanic GH, Briggaman RA, Gammon WR: The epidermolysis bullosa acquisita antigen, a major component of the cutaneous basement membrane is a glycoprotein with collagenous domains. J Invest Dermatol 86:668–672, 1986
14. Paller AS, Queen LL, Woodley DT, Gammon WR, O'Keefe EJ, Briggaman RA: A mouse monoclonal antibody against a newly discovered basement membrane component, the epidermolysis bullosa acquisita antigen. J Invest Dermatol 84:215–217, 1985
15. Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophase T4. Nature (Lond) 227:680–685, 1970
16. Towbin HT, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc Natl Acad Sci USA 76:4350–4354, 1979
17. Woodley DT, Rao CN, Hassell JR, Liotta LA, Martin GR, Kleinman KH: Interactions of basement membrane components. Biochim Biophys Acta 761:278–283, 1983
18. Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK: Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 252:1102–1106, 1977
19. Lecroisey A, Keil B: Differences into degradation of native collagen by two microbial collagenases. Biochem J 179:53–58, 1979.
20. Eisen AZ: Human skin collagenase: localization and distribution in normal human skin. J Invest Dermatol 52:442–448, 1969
21. Gross J, Nagai Y: Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. Proc Natl Acad Sci USA 54:1196–1204, 1965
22. Selzer JL, Adams SA, Grant GA, Eisen AZ: Purification and properties of a gelatin specific neutral protease from human skin. J Biol Chem 256:4662–4668, 1981